

Phylogeny, taxonomy and grouping of *Trypanosoma rangeli* isolates from man, triatomines and sylvatic mammals from widespread geographical origin based on SSU and ITS ribosomal sequences

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SUMMARY

Phylogenetic relationships among *Trypanosoma rangeli* isolates from man, wild mammals and triatomine bugs from widespread geographical origin were inferred by comparison of the small subunit of ribosomal gene sequences. The phylogenetic trees indicated that the subgenus *Herpetosoma* is polyphyletic and strongly supported division of this group into two monophyletic lineages, one made up of *T. rangeli*, *T. rangeli*-like and allied species and other consisting of *T. lewisi* and related taxa. Based on phylogenetic analysis, morphology, behaviour in vertebrate and invertebrate hosts and epidemiology we propose: a) the validation of *Herpetosoma* as a taxon comprised only for species of group *lewisi* and the maintenance of *T. lewisi* as the type-species of this subgenus; b) the classification of *T. rangeli*, *T. rangeli*-like and allied species into a '*T. rangeli*-clade' more closely related to *Schizotrypanum* than to *T. lewisi* or *T. brucei*. The phylogenetic tree disclosed at least 4 groups within the clade *T. rangeli*, all confirmed by polymorphism of the internal transcribed spacer, thus conferring for the first time phylogenetic support to groups of *T. rangeli* and corroborating the high complexity of this taxon. Grouping was independent of their mammalian host-species and geographical origin, indicating that other factors are determining this segregation.

Key words: *Trypanosoma rangeli*, *Herpetosoma*, phylogeny, taxonomy, South American trypanosomes, Brazilian Amazon, polymorphism, *Trypanosoma lewisi*.

INTRODUCTION

Understanding the phylogenetic relationships among trypanosomes from man, domestic and wild mammals is a crucial problem in the evolutionary history of American trypanosomiasis. *Trypanosoma rangeli* and *Trypanosoma cruzi* are the only trypanosomes infecting man in Latin America and sharing vertebrate and invertebrate hosts. Vectors of *T. rangeli* are triatomine bugs, mainly of the genus *Rhodnius*. Like *T. cruzi*, *T. rangeli* also lacks mammalian host specificity and has been reported infecting a variety of mammal species. The animals found naturally infected with *T. rangeli* belong to 5 distinct mammalian orders, which include human and non-human primates, Xenarthra, Didelphi-

morphia, Carnivora and Rodentia (Hoare, 1972; Añez, 1982; D'Alessandro & Saraiva, 1992, 1999; Vallejo *et al.* 2003).

Human infection by *T. rangeli* is highly prevalent in Central and Northwest South America, where this species is sympatric with *T. cruzi* (D'Alessandro & Saraiva, 1999; Vallejo *et al.* 2003). In contrast, only three confirmed human cases were reported in Brazil, all in the Amazon Region (Coura *et al.* 1996), where the presence of triatomines and sylvatic mammals infected with *T. rangeli* are common (Deane *et al.* 1972; Miles *et al.* 1983; Ziccardi *et al.* 2000; Maia da Silva *et al.* 2004). Outside Amazonia, *T. rangeli* was only reported in sylvatic mammals and triatomines of Southern and Southeast Brazilian regions (Steindel *et al.* 1991; Ramirez *et al.* 1998).

Despite sharing hosts, *T. rangeli* and *T. cruzi* are remarkably different with respect to their behaviour in both invertebrates and vertebrates. In contrast to *T. cruzi*, *T. rangeli* may be harmful or even lethal to its vector, inducing failures to moult,

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body abnormalities and reduced ability (or even the inability) of the insect to feed (Añez, 1984; D'Alessandro & Saraiva, 1992, 1999). In contrast to *T. cruzi*, *T. rangeli* is harmless to its mammalian hosts. The parasitaemia is generally very low and may persist for a long time (Hoare, 1972; D'Alessandro & Saraiva, 1992, 1999). The infection produced by *T. rangeli* is poorly understood and nothing definitive is known about the mode and the site of reproduction. Dividing blood trypomastigotes and intracellular tissue forms, such as amastigotes typical of *Schizotrypanum* species, have not been confirmed in vertebrate hosts of *T. rangeli* (Añez, 1981, 1982; D'Alessandro & Saraiva, 1992, 1999; Eger-Mangrich *et al.* 2001).

Traditionally, *T. rangeli* is classified in the subgenus *Herpetosoma* of *Stercoraria* together with *T. lewisi*, which is the type species of this subgenus. Two sections without taxonomic status were created by Hoare (1972) to separate mammalian trypanosomes whose development occurs exclusively in the gut of vectors and which are transmitted by faeces (*Stercoraria*) from trypanosomes transmitted by inoculation of metacyclic forms in the saliva of tsetse fleas (*Salivaria*). The life-cycle of *T. rangeli* in the triatomine vectors has been reported to have features of both the stercorarian and the salivarian trypanosome life-cycles. Although *T. rangeli* develops in the insect's gut, trypanosomes in faecal material are not metacyclic forms (Tobie, 1964; Añez, 1983*a, b*) and faecal contamination is not the usual mechanism of transmission (D'Alessandro & Saraiva, 1999). After initial development in the gut, the flagellates penetrate through the gut wall, a route peculiar to this species, and develop in the haemolymph and salivary glands. The triatomine-bugs transmit *T. rangeli* by inoculation of saliva containing metacyclic forms during feeding or probing (Tobie, 1970; Añez, 1983*a, b*; Hecker, Schwarzenbach & Rudin, 1990).

On account of this unusual combination of transmission routes and life-cycle features, producing infective forms in both the posterior and anterior stations of the vector, the systematic position of *T. rangeli* has been a subject of controversy. Hoare (1972) considered *T. rangeli* an aberrant species of the subgenus *Herpetosoma*. Añez (1982) proposed removing *T. rangeli* from the subgenus *Herpetosoma*, creating the subgenus *Tejeraia* within *Salivaria*. Initial molecular analyses only served to increase the controversy surrounding the evolutionary relationships of *T. rangeli* because data from a few isolates about sequences of β -tubulin (Amorim, Momen & Traub-Cseko, 1993) and cysteine-protease (Tanaka *et al.* 1994) genes, and karyotyping (Henriksson *et al.* 1996) also suggested that *T. rangeli* is more related to *T. brucei* than to *T. cruzi*. However, D'Alessandro & Saraiva (1999) supported its maintenance within *Stercoraria* as a *Herpetosoma* species while Stevens & Gibson (1999), in a broad phylogenetic analysis

of SSU rRNA sequences, positioned 5 isolates of *T. rangeli* within *Stercoraria* and more close to *Schizotrypanum* spp. than to all other trypanosomes, thus indicating that the taxonomic position of *T. rangeli* and the taxonomy of the whole *Herpetosoma* must to be reviewed (Stevens & Gibson, 1999; Stevens *et al.* 1999*a*, 2001).

Polymorphism among *T. rangeli* populations was shown by: zymodemes (Miles *et al.* 1983; Steindel *et al.* 1994), DNA fingerprinting (Macedo *et al.* 1993), mini-exon gene sequences (Grisard, Campbell & Romanha, 1999), karyotyping (Henriksson *et al.* 1996) and random amplification of polymorphic DNA (RAPD) (Steindel *et al.* 1994). According to these studies the genetic polymorphism among isolates from different geographical origins permitted their partition into 2 groups. We recently identified at least 4 distinct genetic lineages among a large number of isolates by RAPD analysis, thus revealing that the complexity of *T. rangeli* is higher than previously described (Maia da Silva *et al.* 2004). While the taxonomic position of *T. rangeli* from man and triatomines is now relatively well established, trypanosomes from sylvatic mammals classified as *T. rangeli*, *T. rangeli*-like and allied species were under-represented in the previous studies and the little available data suggested that reclassification of some isolates may be prudent (Stevens & Gibson, 1999; Stevens *et al.* 1999*a, b*, 2001; Maia da Silva *et al.* 2004). Due to the genetic variability within *T. rangeli* and considering that, besides the biased selection for human and insect isolates, previous studies included few isolates representative of all distinct genetic groups of *T. rangeli*, we do not have sufficient data yet to define the evolutionary relationships among *T. rangeli* isolates and allied species. Study of several isolates from distinct host species and geographical origin is required to evaluate the segregation of *T. rangeli* into lineages, their evolutionary relationships and thus, their taxonomic status. With these points in mind, we investigated the phylogenetic relationships based on polymorphism in SSU and ITS rDNA sequences of 34 isolates of *T. rangeli* from man, triatomine bugs, non-human primates and other sylvatic mammals (opossum, anteater, sloth, rodent) from a widespread geographical region.

MATERIALS AND METHODS

T. rangeli isolates and other trypanosomes

In this study we used 34 isolates of *T. rangeli* and allied species from distinct hosts and geographical origin, embracing widespread geographical origins from Central and South Americas. From Brazil, we analysed isolates from 5 States from northern (Amazonia Region) and southern (Santa Catarina State) regions (Fig. 1, Table 1). Isolation, culturing



Fig. 1. Geographical origin of *Trypanosoma rangeli* isolates and allied species employed in this study. Groups of *T. rangeli* defined in this study: A (■), B (●), C (◆) and D (*).

and classification of all new isolates were done as previously described (Maia da Silva *et al.* 2004). Trypanosomes used in this study were tested using 2 *T. rangeli*-specific PCR assays (Vargas *et al.* 2000; Maia da Silva *et al.* 2004). Other trypanosome species used in this study were cultivated in LIT medium at 28 °C (Table 1). Trypanosomes are cryopreserved in the Trypanosomatid Culture Collection (TCC) of the Department of Parasitology of the University of São Paulo, SP, Brazil.

PCR amplification, analysis of restriction polymorphism and sequencing of SSU rRNA sequence

DNA of trypanosomes obtained by the phenol-chloroform extraction method was used as template for PCR amplification of a 900 bp DNA fragment containing partial SSU rRNA sequence using primers 609F and 706R (Fig. 2). Amplifications were done in reactions of 50 µl containing 100 ng of DNA, 2.5 U of *Taq* DNA polymerase, 0.2 mM each dNTP and 200 pM of primers. The reactions were cycled 30 times as follows: 1 min at 94 °C, 2 min at 48 °C and 1 min at 72 °C. Amplification products of 3–5 separated PCR reactions were purified by Spin-X (Costar), pooled and automatically sequenced by primer walking in both directions. For restriction analysis, amplified DNA was digested with *Rsa* I (Biolabs) enzyme, analysed on 2.0% agarose gels and stained with ethidium bromide. Polymorphism of restriction enzyme sites on the aligned SSU rRNA sequences of *T. rangeli* isolates was investigated using the Clone program (CLONE

MANAGER version 3.11; www.sciencedirect.com/science/article/pii/S0950268804005931).

PCR amplification, Southern blot hybridization analysis and sequencing of ITS ribosomal gene

DNA fragments corresponding to ITS1, 5.8S and ITS2 sequences of the ribosomal gene were amplified using primers IR1 and IR2 (Cupolillo *et al.* 1995). For exclusive amplification of ITS1 sequences the primers IR1 and 5.8R (Fig. 2) were used as described by Cupolillo *et al.* (1995). Amplified products were electrophoresed on 2% agarose gels and transferred to nylon membranes (Hybond-N, Amersham Pharmacia). Membranes were hybridized with amplified fragment generated from DNA of *T. rangeli* isolates, labelled by random primed synthesis with [α -³²P] dCTP (Ready to Go kit, Amersham Pharmacia). Southern blots were pre-hybridized for 1 h and hybridized, at 43 °C, for 16–18 h in 2 × SSC, 2% SDS, 4.0 mM sodium pyrophosphate and 40 mg/ml of salmon sperm DNA. Membranes were washed 3 times for 15 min each in 0.1 × SSC, 2% SDS and 4 mM sodium pyrophosphate at 55 °C. Amplified ITS1 sequences of selected isolates were separated in 2% agarose gels, stained with ethidium bromide, excised from the gels, purified by Spin-X (Costar) and cloned (pGEM kit, Amersham Pharmacia). Sequences of 2–3 clones from each isolate were determined by automated sequencing.

Alignment of SSU and ITS1 ribosomal sequences and phylogenetic inference

SSU rRNA (V7-V8) sequences from 27 isolates of *T. rangeli* and allied species, from 3 new isolates of *T. cruzi* from Amazonia (2 from monkeys and 1 from man), and from *T. blanchardi* and *T. rabinowitschae* were determined in this study and aligned with sequences from GeneBank (Table 1). Alignments were made manually using as guide the general alignment in the rRNA database (<http://rrna.uia.ac.be/>). Phylogenies were inferred using both Maximum Parsimony (MP) and Maximum-Likelihood (ML) analysis. MP analysis was done as before (Stevens *et al.* 1999b). ML parameters were optimized using the hierarchical likelihood test in Modeltest 3.06 (Posada & Crandall, 1998) and estimates using TreePuzzle 5.0 (Strimmer & Von Haeseler, 1996). Distance matrix was calculated using Neighbour Joining (Saitou & Nei, 1987) with parameters from the Tamura & Nei substitution model. Similarity matrix was performed using Point Replacer v2.0 (<http://www.geocities.com/alvesjmp/software.html>). Bootstrap analyses with 100 replicates were performed using PAUP 4.0b10. The trees were rooted using *Trypanoplasma borreli* as outgroup for *Trypanosoma*.

Table 1. *Trypanosoma rangeli* isolates and allied species and trypanosome species of *Trypanosoma lewisi* group used in this study showing host species and geographical origins

TCC	Trypanosome	Host species	Origin	Accession no.	
				SSU rRNA	ITS rDNA
<i>T. rangeli</i> isolates					
031	S. Augustin	human	<i>Homo sapiens</i>	Colombia	AJ012417
020	Macias	human	<i>Homo sapiens</i>	Venezuela	AJ012415
014	PG ^a	human	<i>Homo sapiens</i>	Panama	AJ012416
—	RGB	dog	<i>Canis familiaris</i>	Colombia	AJ009160
021	Choachi	triatomine	<i>Rhodnius prolixus</i>	Colombia	AJ012414
328	1625 ^a	human	<i>Homo sapiens</i>	El Salvador	AY491738
022	Palma-2	triatomine	<i>Rhodnius prolixus</i>	Venezuela	AY491741
023	SC58	rodent	<i>Echimys dasythrix</i>	Brazil (SC)	AY491745
024	H8GS	human	<i>Homo sapiens</i>	Honduras	AY491744
—	H9	human	<i>Homo sapiens</i>	Honduras	AY491744
591	SMH-03 ^b	human	<i>Homo sapiens</i>	Guatemala	AY491739
594	SMH-79 ^b	human	<i>Homo sapiens</i>	Guatemala	AY491740
529	MHOM/VE/99/ CH-99 ^c	human	<i>Homo sapiens</i>	Venezuela	AY491742
534	MAN/VE/00/ LOBITA-00 ^c	dog	<i>Canis familiaris</i>	Venezuela	AY491743
086	AM80 ^d	human	<i>Homo sapiens</i>	Brazil (AM) Rio Negro	AY491766
261	AM11 ^d	human	<i>Homo sapiens</i>	Brazil (AM) Rio Negro	AY491758
220	AT-AEI	monkey	<i>Saimiri sciureus</i>	Brazil (PA) Marajó Island	AY491747
202	AT-ADS	monkey	<i>Saimiri sciureus</i>	Brazil (PA) Marajó Island	AY491746
207	AE-AAA	monkey	<i>Cebuella pygmaea</i>	Brazil (AC) Rio Branco	AY491752
194	AE-AAB	monkey	<i>Cebuella pygmaea</i>	Brazil (AC) Rio Branco	AY491753
233	4-30	monkey	<i>Saguinus labiatus labiatus</i>	Brazil (AC) Rio Branco	AY491756
238	5-31	monkey	<i>Saguinus labiatus labiatus</i>	Brazil (AC) Rio Branco	AY491754
236	8-34	monkey	<i>Saguinus fuscicollis weddelli</i>	Brazil (AC) Rio Branco	AY491755
205	M-12229 ^e	monkey	<i>Aotus</i> sp	Brazil (AM) Manaus	AY491757
353	Maloch-05	monkey	<i>Callicebus maloch cripeus</i>	Brazil (AC) P. de Castro	AY491750
369	ROma 01	opossum	<i>Didelphis marsupialis</i>	Brazil (RO) Monte Negro	AY491748
382	ROma 06	opossum	<i>Didelphis marsupialis</i>	Brazil (AM) Rio Negro	AY491749
416	M 2495	monkey	<i>Alouatta stramineus</i>	Brazil (RO) Monte Negro	AY491760
427	M 2570	monkey	<i>Callicebus lugens</i>	Brazil (AM) Rio Negro	AY491751
<i>T. rangeli</i> allied species					
012	<i>T. saimiri</i> (M12312) ^e	monkey	<i>Saimiri sciureus</i>	Brazil (AM) Manaus	AY491768
013	<i>T. preguici</i> (M15177) ^e	sloth	<i>Choloepus didactylus</i>	Brazil (PA) Belém	AY491767
010	<i>T. legeri</i> (M11680) ^e	anteater	<i>Tamandua tetradactyla</i>	Brazil (PA) Belém	AY491769
032	<i>T. legeri</i> (M11683) ^e	anteater	<i>Tamandua tetradactyla</i>	Brazil (PA) Belém	AY491759
	<i>T. minasense</i>	monkey	<i>Saimiri boliviensis</i>		AJ012413
	<i>T. leuvenhoekei</i>	sloth	<i>Choloepus didactylus</i>	Panama	AJ012412
<i>T. lewisi</i> group					
	<i>T. lewisi</i>	rodent	<i>Rattus</i> sp	England	AJ009156
	<i>T. lewisi</i>	rodent	<i>Rattus</i> sp	England	AJ223566
	<i>T. musculi</i>	rodent	<i>Mus musculus</i>	—	AJ223568
061	<i>T. blanchardi</i> ^f	rodent	<i>Eliomys quercinus</i>	France	AY491764
059	<i>T. rabinowitzschae</i> ^f	rodent	<i>Cricetus cricetus</i>	France	AY491765
	<i>T. microti</i>	rodent	<i>Microtus agrestis</i>	England	AJ009158

Source of trypanosomes were: Trypanosomatid Culture Collection (TCC), USP, SP, Brazil; Institute of Tropical Medicine, São Paulo; SP, Brazil; Centro de Pesquisa René Rachou, MG, Brazil; University of Liverpool, Liverpool, UK. ^a Sousa, O.; ^b Rodas, A.; ^c Añez, N.; ^d Coura, J. R.; ^e Shaw, J. J.; ^f Noyes, H.

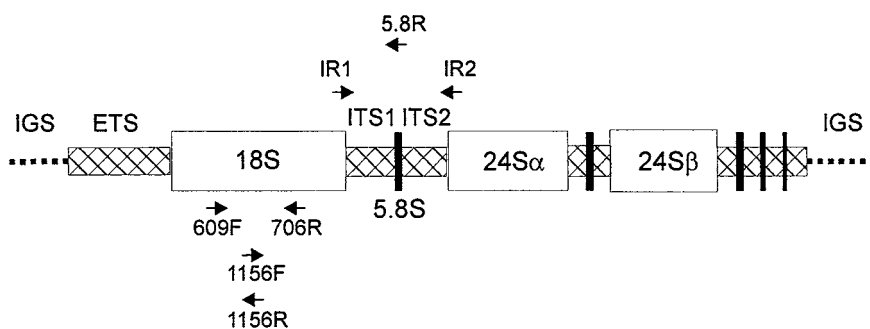
The ITS1 sequences determined in this study were aligned with others from GeneBank (Table 1). Alignments of our sequences did not reveal significant polymorphism among sequences from different clones of the same isolates. Phylogenetic analyses were performed as described above for SSU rRNA sequences.

RESULTS

Origin and identity of *T. rangeli* isolates

Ten isolates of *T. rangeli* from man, 2 from triatomines, 13 from non-human primates of Brazilian

Amazon, 7 from other wild mammals and 2 from dogs were used in this study. These isolates came from 7 countries, from Guatemala to Brazil (Fig. 1). All new isolates of *T. rangeli* investigated in this study showed all traditional taxonomic criteria (morphology and behaviour in mice and triatomines) compatible with *T. rangeli* (Maia da Silva *et al.* 2004). In addition, all *T. rangeli* isolates were confirmed by PCR assays developed to detect specifically this species. Both methods employed amplified DNA fragments of identical length for all isolates, independently of their genetic groups (Vargas *et al.* 2000; Maia da Silva *et al.* 2004).



5' → 3'	
609F	- CACCCGCGGTAATTCCAGC
706R	- TTGAGGTTACAGTCTCAG
1156F	- CGTACTGGTGCGTCAGAGG
1156R	- CCTCTGACGCACCAAGTACG
IR1	- GCTGTAGGTGAACCTGCAGCAGCTGGATCATT
IR2	- GCGGGTAGTCCTGCCAAACACTCAGGTCTG
5.8R	- GGAAGCCAAGTCATCCATC

Fig. 2. Schematic diagram of the ribosomal gene showing the annealing sites for oligonucleotides (in box) used for PCR amplifications and DNA sequencing.

Phylogenetic relationship and taxonomic position of *T. rangeli* isolates based on SSU rRNA sequences

Phylogenetic relationships were inferred from comparison of variable regions (V7-V8) of SSU rRNA sequences among 34 isolates of *T. rangeli* and allied species, including 5 previously published sequences and 27 sequenced in this study. Nineteen sequences of other trypanosome species were also included (5 obtained in this study) (Table 1). Similar strongly supported branching patterns were obtained using both MP (data not shown) and ML (Fig. 3A) methods, as well as using different data sets varying in species, number of samples and aligned sequences (only V7 or V7-V8 for all isolates and V7-V9 for few isolates).

According to the topology of our phylogenetic tree, all isolates of *T. rangeli* and allied species form a strongly supported monophyletic assemblage (clade *T. rangeli*), which always clustered together (with 100% of bootstrap support). This clade was characterized by a low inter- and intra-specific sequence divergence (maximum of 2.1% and an average of 0.9%), demonstrating that organisms within this clade are highly genetically related. The closest relative to the *T. rangeli* clade was *T. cruzi* (~20% of sequence divergence) and other species of the subgenus *Schizotrypanum* (*T. c. marinkellei* and *T. dionisii*). We also included in the phylogenetic analysis isolates of *T. cruzi* belonging to the major phylogenetic lineages (*T. cruzi* I, *T. cruzi* II and Z3), including new isolates from man and from non-human primates of the Brazilian Amazon (Table 1). Contrasting with the high homogeneity within the clade *T. rangeli*, significant intra-specific hetero-

geneity was detected among lineages of *T. cruzi* (4.5% of sequence divergence) and in the subgenus *Schizotrypanum*, including *T. cruzi*, *T. c. marinkellei* and *T. dionisii* (7.8% of divergence).

T. lewisi and allied species (*T. musculi*, *T. microti*, *T. blanchardi* and *T. rabinowitschae*) from different host-species and geographical regions were tightly clustered together (100% of bootstrap) into the *T. lewisi* clade. Species of the *T. lewisi* clade showed a high intra- and inter-specific sequence similarity (95%), similar to that within the *T. rangeli* clade. Despite sequence divergence (~20%) separating clades, *T. rangeli* and *T. cruzi* showed similarity to the divergence (~20%) between *T. rangeli* and *T. lewisi* clades, clade *T. lewisi* was positioned as an outgroup of the branch containing *T. cruzi* and *T. rangeli* in all inferred trees (100% of replicates). Similarly, *T. rangeli* and *T. brucei* were separated by a high genetic distance (~35% of sequence divergence) strongly supported (100% of bootstrap) in all inferred trees (Fig. 3A).

Subgroups of *T. rangeli* defined by polymorphism on the SSU rRNA sequences

Despite the high similarity among V7-V8 rRNA sequences from all members of the clade *T. rangeli* (99.2% of similarity), phylogenetic analysis based on these sequences segregated these organisms into 4 groups: A, B, C and D (Fig. 2, Table 2). The same branching pattern was observed in all inferred phylogenetic trees. In agreement with tree topology, analysis of the matrix of pairwise differences between SSU rRNA sequences (which provided a measure of absolute genetic differences between taxa) showed

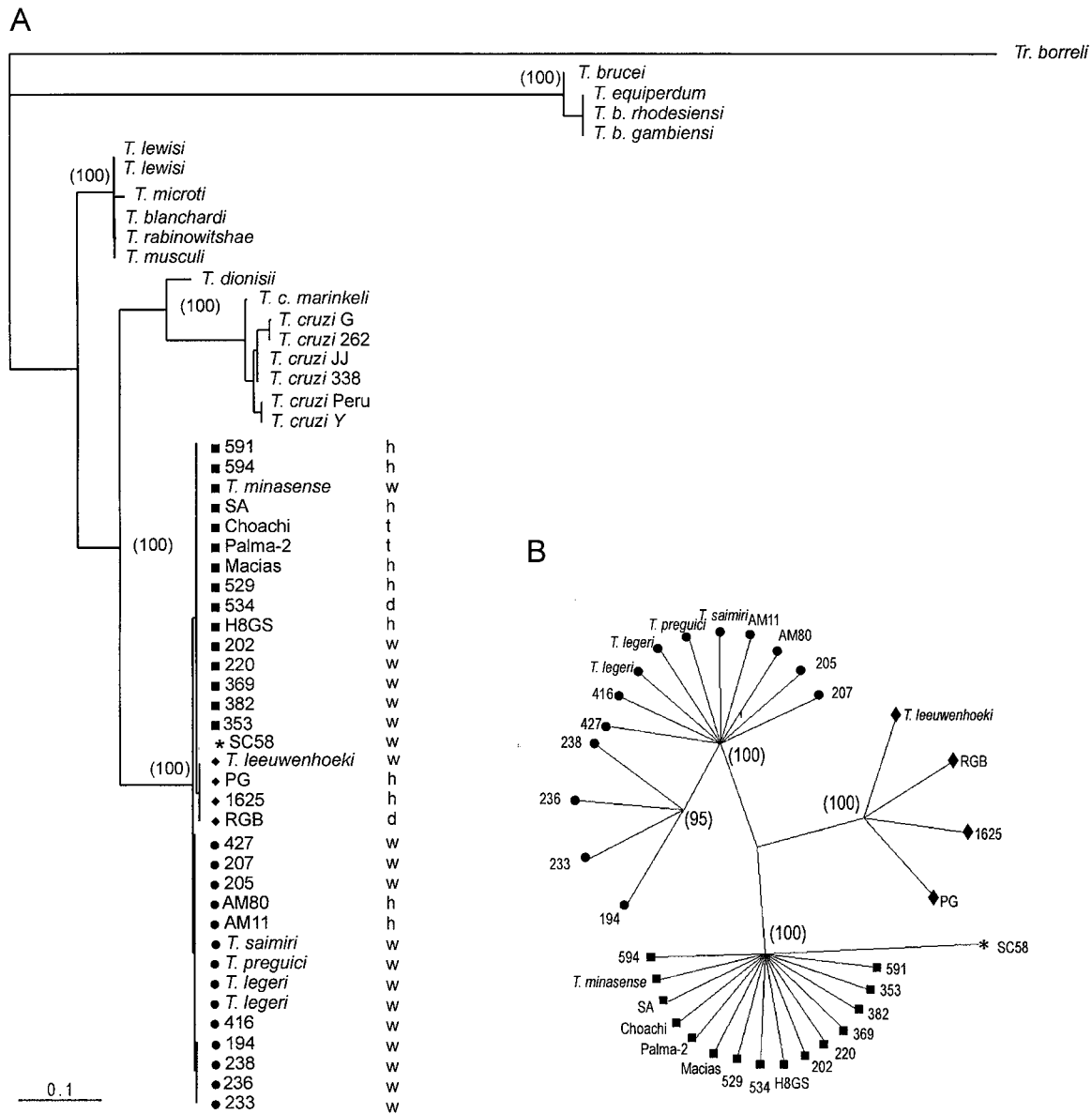


Fig. 3. (A) Phylogenetic tree based on bootstrapped Maximum Likelihood analysis of SSU (V7-V8) rRNA sequences from 52 trypanosomes. (B) Dendrogram of 34 *Trypanosoma rangeli* isolates and allied species constructed by Maximum Likelihood analysis of V7-V8 SSU rRNA sequences. The numbers in parenthesis refer to the bootstrap values of the clusters in 100 replicates. *T. rangeli* groups: A (■), B (●), C (◆) and D (*). Source of trypanosomes: h, human; w, wild mammals; t, triatomine bugs; d, dog.

significant genetic polymorphism between distinct groups within the clade *T. rangeli*. According to polymorphism on SSU rRNA sequences, organisms were distributed as follows: Group A, composed of all isolates from Colombia, Venezuela, Honduras and Brazilian isolates from Rondônia and Pará (Marajó Island) States; Group B, formed exclusively by Brazilian isolates from Acre, Amazonas (Manaus and Alto e Médio Rio Negro) and Pará (Belém) States; Group C, constituted by isolates from Panama, El Salvador and Colombia (Fig. 3); Group D, represented in this study exclusively by the isolate SC58, from Santa Catarina State, Southern Brazil, was segregated into a distinct branch, although it clustered close to group A, separated by 1.1% of

sequence divergence. Little or no variability was observed within groups: Groups A, C and D were more related to each other (0.7–1.0% of divergence) than to group B (~2.1% of divergence from the other groups). Group B was divided into subclades B and B', the last composed by isolates from Acre sharing identical sequences with small sequence divergence (0.2%) from the rest of the isolates of Group B (Figs 2, 3).

PCR-RFLP of SSU as a tool for genotyping subgroups of T. rangeli

Aiming to standardize a simple and quick method to type any unknown *T. rangeli* isolates in

Table 2. Grouping of *Trypanosoma rangeli* isolates and allied species based on RAPD, SSU rRNA and ITS analysis

TCC	Trypanosome	Grouping based on:				
		RAPD ^a	Ribosomal genes			
			SSU (PCR-RFLP)	SSU (V7-V8) ^b	ITS1 + 5.8S + ITS2 (Length in kb) ^c	ITS 1 (Length in bp) ^c
031	S. Augustin	A	A	A	A (1.3)	A (415)
020	Macias	A	A	A	A (1.3)	A (415) ^d
021	Choachi	A	A	A	A (1.3)	A (415) ^d
022	Palma-2	A	A	A	A (1.3)	A (415) ^d
024	H8GS	A	A	A	A (1.3)	A (415)
	H9	N.D.	N.D.	N.D.	N.D.	A (415) ^d
591	SMH-03	A	A	A	A (1.3)	A (415)
594	SMH-79	A	A	A	A (1.3)	A (415)
529	M HOM/VE/99/CH-99	A	A	A	A (1.3)	A (415)
534	M AN/VE/00/LOBITA-00	A	A	A	A (1.3)	A (415)
220	AT-AEI	A	A	A	A (1.3)	A (415)
202	AT-ADS	A	A	A	A (1.3)	A (415)
353	Maloch-05	A	A	A	A (1.3)	A (415)
369	ROma 01	A	A	A	A (1.3)	A (415)
382	ROma 06	A	A	A	A (1.3)	A (415)
	<i>T. minasense</i>	N.D.	N.D.	A	N.D.	N.D.
	<i>T. leeuwenhoekii</i>	N.D.	N.D.	C	N.D.	N.D.
014	PG	C	C	C	C (1.2)	C (425) ^d
328	1625	C	C	C	C (1.2)	C (425) ^d
023	SC58	D	A	D	D (1.4)	D (440) ^d
086	AM80	B	B	B	B (1.1)	B (340) ^d
261	AM11	B	B	B	B (1.1)	B (340) ^d
207	AE-AAA	B	B	B	B (1.1)	B (340)
194	AE-AAB	B	B	B'	B (1.1)	B (340)
233	4-30	B	B	B'	B (1.1)	B (340)
238	5-31	B	B	B'	B (1.1)	B (340)
236	8-34	B	B	B'	B (1.1)	B (340)
205	M-12229	B	B	B	B (1.1)	B (340)
416	2495	B	B	B'	B (1.1)	B (340)
427	2570	B	B	B'	B (1.1)	B (340)
012	<i>T. saimiri</i>	B	B	B'	B (1.1)	B (340) ^d
013	<i>T. preguici</i>	B	B	B'	B (1.1)	B (340)
010	<i>T. legeri</i>	B	B	B'	B (1.1)	B (340)
032	<i>T. legeri</i>	B	B	B	B (1.1)	B (340)

^a Grouping based on RAPD analysis previously published or determined as before (Maia da Silva *et al.* 2004).

^b Groups derived from the phylogenetic tree constructed using V7-V8 SSU rRNA sequences (Fig. 3).

^c Approximated length of ITS rDNA gene sequences determined by electrophoresis in agarose gels (Figs 5, 6).

^d Groups defined by branching pattern of the dendrogram based on ITS1 sequences (Fig. 6).

groups dispensing SSU rRNA DNA sequencing, PCR-amplified fragments of this gene were digested with *Rsa* I restriction enzyme. This enzyme was selected after analysis of aligned sequences had detected restriction sites within the amplified fragments capable of generating different fragment patterns according to the groups of isolates. Restriction analysis of SSU rRNA amplified fragments from *T. rangeli* isolates disclosed three major patterns of DNA fragments in agarose gels, corresponding to the three major groups of *T. rangeli* (A, B and C) defined in the phylogenetic trees (Fig. 3A). PCR-RFLP patterns generated by digestion with *Rsa* I enzyme did not distinguish Group D (isolate SC58) from Group A (Fig. 4). Additional analysis with other enzymes can be used for this purpose.

Analysis of polymorphism of the internal transcribed spacer (ITS) of ribosomal gene

In order to clarify the grouping patterns within the clade *T. rangeli*, we have evaluated the polymorphism among *T. rangeli* isolates using the more divergent sequences of ITS ribosomal gene. For this purpose, we initially evaluated the polymorphism by comparing length and sequence of the PCR-amplified DNA fragments containing the whole ITS (ITS1, 5.8S and ITS2) from DNA of 11 isolates (Fig. 5A). Results showed length differences that permitted us to distribute the isolates into the same groups defined by the tree branching pattern (Fig. 3A). The degrees of conservation of ITS sequences among members of distinct groups of *T. rangeli* were evaluated by

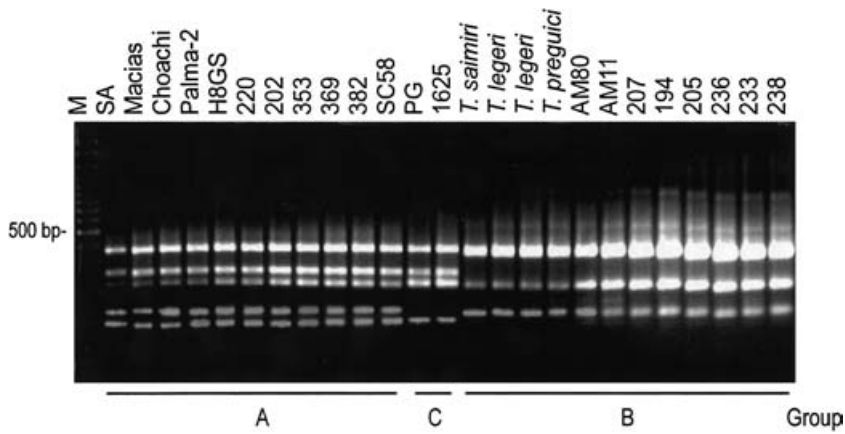


Fig. 4. PCR-RFLP analysis as a tool for quick genotyping of *Trypanosoma rangeli*. Agarose gel (2%) of the restriction DNA fragments generated by digestion of amplified V7-V8 regions of SSU rRNA with *Rsa* I enzyme and stained with ethidium bromide.

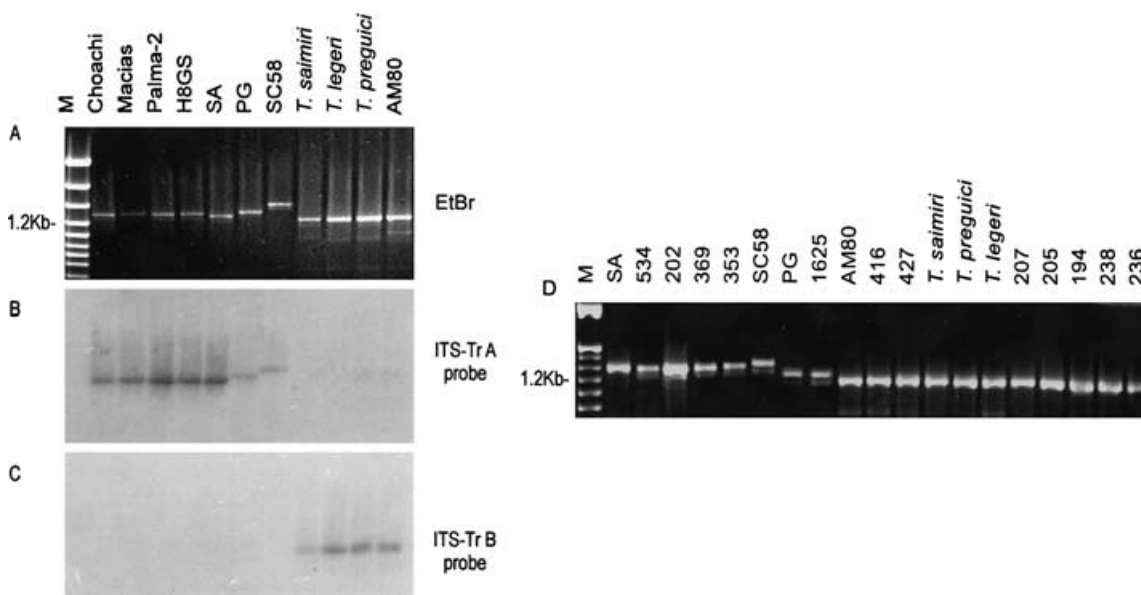


Fig. 5. Agarose gel (2%) showing amplified fragments of whole ITS (ITS1 + 5.8S + ITS2) from *Trypanosoma rangeli* isolates and allied species stained with ethidium bromide (A, D). Southern blotting of the gel in (A) hybridized with probes constituted by amplified ITS sequences of *T. rangeli* isolates: ITS-TrA from SA (B) or ITS-TrB from AM80 (C).

cross-hybridization analysis (Fig. 1B,C). For this purpose, the ITS amplified fragments were analysed by Southern blot hybridization using probes TrA and TrB, which are constituted by the amplified fragments from DNA of *T. rangeli* SA and AM80, respectively. These isolates were selected because they represent the 2 major groups of isolates from the Brazilian Amazon. Results revealed that each probe cross-hybridized only with organisms belonging to groups from which they were derived, with no cross-hybridization between isolates from groups A and B. Very faint signals, and only when hybridized with probe TrA, were detected for isolates SC58 (Group D) and PG (Group C) (Fig. 5B) and thus are in agreement with tree topology, in which both isolates seemed to be more related to group A than to group B. The polymorphism of whole ITS was assessed for

12 more isolates (Fig. 5D) and, according to the length of the amplified fragments, the 23 isolates examined were distributed into the same groups shown in the dendrogram (Table 2). Thus, this approach provided a quick and simple tool to type *T. rangeli* in groups.

Despite significant length and sequence polymorphism of ribosomal sequences among *T. rangeli* isolates, results showed higher conservation among isolates of the same groups than between organisms belonging to different groups. Corroborating these data, analysis of *T. rangeli* ITS sequences showed that both ITS1 and ITS2 are highly divergent among isolates of this species, whereas the 5.8S gene was identical. In order to compare sequences presenting different evolutionary rates, we compared data from the highly conserved SSU with that based

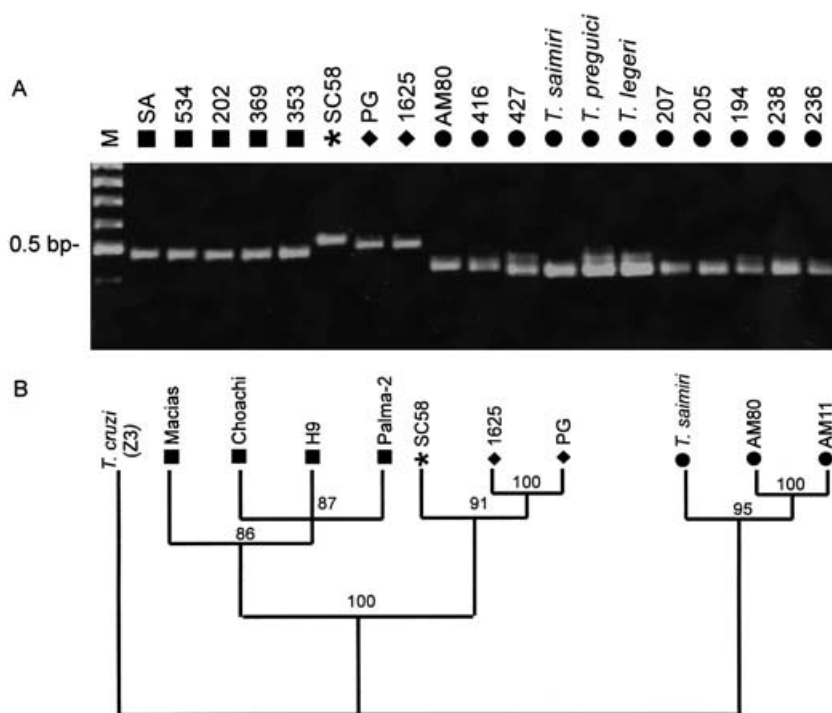


Fig. 6. (A) Agarose gel (2%) showing amplified fragments of ITS1 from *Trypanosoma rangeli* isolates and allied species stained with ethidium bromide. (B) Dendrogram representing the genetic relatedness among 7 *T. rangeli* isolates and *T. saimiri* constructed by maximum likelihood analysis of ITS1 rRNA sequences. The numbers in parenthesis refers to the bootstrap values of the clusters in 100 replicates. *T. rangeli* groups: A (■), B (●), C (◆) and D (*).

on the more variable ITS1 sequences. Length polymorphism of amplified ITS1 from 19 isolates permitted the same grouping originated by whole ITS, despite small heterogeneity within the group B (Fig. 6A, Table 2). For comparative analysis, sequences of isolates of Group B (*T. saimiri* and AM80), Group C (PG and 1625) and Group D (SC58) were aligned with sequences of *T. rangeli* from Genbank (isolates Macias, Choachi, H9, Palma-2 and AM11), aiming to obtain a good set of sequences containing members of all major groups of *T. rangeli* (Fig. 3; Table 2). The genetic relatedness among the isolates assessed through a matrix of pairwise divergence showed high sequence divergence between groups A and B of *T. rangeli*. Contrasting with the low divergence among SSU rRNA sequences (1.2%) these groups were separated by 39.4% of ITS1 sequence divergence. The topology of the dendrogram based on ITS1 sequences disclosed a branching pattern in concordance with clustering on an SSU rRNA-based tree (Fig. 6B).

DISCUSSION

The genus *Trypanosoma* seems to be among the most diverse lineage within the Trypanosomatidae family and its monophyly has been supported by several studies (Haag, O'h Uigin & Overath, 1998; Hannaert, Opperdoes & Michels, 1998; Stevens & Gibson, 1999; Stevens *et al.* 1999b, 2001). Molecular phylogenetic studies supported the division of

mammalian trypanosomes into 2 major groups, which are represented by stercorarian or salivarian trypanosomes, separated by a very large genetic distance and constituted by highly divergent subgenera. Since the classification of *Trypanosoma* proposed by Hoare (1972), the taxonomy of this genus has remained remarkably stable despite a lot of data pointing towards the need for an urgent revision. Nowadays, of the three subgenera of Stercoraria, only *Schizotrypanum* appears to be a monophyletic group, whereas *Herpetosoma* and *Megatrypanum* were shown to be polyphyletic in studies based on SSU rRNA (Stevens & Gibson, 1999; Stevens *et al.* 1999, 2001). In fact, Hoare (1964) had already pointed out that not all stercorarian trypanosomes could be fitted with certainty into subgenera, especially in *Megatrypanum* and *Herpetosoma*. Phylogenetic studies pointed to a higher genetic diversity within *Trypanosoma* than considered by the current classification, questioning the whole taxonomy of this genus. Thus, how this genus should be organized in subgenera, species and subspecies is still controversial. It seems probable that continued studies on species within the genus may require the subdivision of the trypanosomes into additional subgenera and epidemiologically relevant groups as well as the creation of several new species.

This study focused on understanding the evolutionary relationships and taxonomic position of *T. rangeli* by inferring phylogenetic relationships among 34 isolates of *T. rangeli*, *T. rangeli*-like and

allied species (*T. saimiri*, *T. preguici*, *T. leeuwenhoekii*) and isolates previously classified as *Megatrypanum* that have been revealed to be *T. rangeli* (*T. minasense* and *T. legeri*) (Stevens *et al.* 1999*a*; Maia da Silva *et al.* 2004). Aiming to infer relationships within *Herpetosoma*, we also analysed 2 *T. lewisi* isolates and 4 allied species. The inferred trees confirmed that subgenus *Herpetosoma* is a strongly supported polyphyletic group divided in 2 distant monophyletic clades, allied to either *T. rangeli* or to *T. lewisi*. Several trypanosome isolates can be readily ascribed to either one of these clades according to traditional taxonomic criteria, as well as by robust molecular methods, enough to propose a reliable taxonomic revision of *Herpetosoma* as previously proposed (Stevens & Gibson, 1999; Stevens *et al.* 1999*a*, 2001). Molecular data are in agreement with the large differences existing among *T. rangeli*, *T. cruzi*, *T. lewisi* and *T. brucei* regarding several aspects such as their life-cycles, vectors, transmission routes, and relationships in both vertebrate and invertebrate hosts. All these species could be clearly distinguished from each other by a number of morphological, biological, and biochemical peculiarities (autapomorphies).

T. lewisi and allied species tightly clustered into a well-supported monophyletic clade, thus justifying the continued recognition of the taxon *Herpetosoma* and the maintenance of *T. lewisi* as the type species of this subgenus, as stated when Hoare (1964, 1972) created this subgenus. Besides the historical priority, this species presents all major features of *Herpetosoma* as originally described (Hoare, 1964, 1972; Molyneux, 1976; Añez, 1982).

T. rangeli and allied species were positioned with stercorarian trypanosomes into a different clade (Clade *T. rangeli*) distinct from the *T. lewisi* clade and close to taxa classically recognized as *Schizotrypanum* (see also Stevens *et al.* 1999*b*, 2001). *T. rangeli* and associated taxa share the following major features: (a) wide mammalian host range; (b) geographical distribution from Central to South America; (c) lack of pathogenicity and low parasitaemia in the vertebrate hosts; (d) pathogenicity to triatomine vectors. However, the only exclusive taxonomic parameter for recognizing trypanosomes as a member of this subgenus is the demonstration of metacyclic trypomastigotes in the salivary glands of triatomine bugs. However, the successful establishment of the extra-intestinal cycle of development of *T. rangeli* depends upon several factors such as vector species, age of isolates maintained in cultures and the method used for bug infection (intracoelomic inoculation or xenodiagnosis) (D'Alessandro & Saraiva, 1992; Maia da Silva *et al.* 2004).

Our phylogenetic analysis also confirmed that *T. rangeli* is more closely related to clade *T. cruzi*, which includes the *Schizotrypanum* species (*T. cruzi*, *T. cruzi marinkellei* and *T. dionisii*) than to all other

trypanosome species. Based on phylogenetic analysis and paleographic evidence, Stevens *et al.* (1999, 2001) hypothesized that *T. rangeli*, *T. cruzi* and a trypanosome from kangaroo diverged from a common ancestor, probably a trypanosome in marsupials on a southern super-continent of South America, Antarctica and Australia. However, divergence on SSU rRNA sequences between *T. rangeli* and *T. cruzi* suggested distinct evolutionary pathways of these species within the Americas. In addition, although sharing geographical distribution, mammalian host range and transmission by triatomines, *T. rangeli* and *T. cruzi* differ significantly in most morphological and biological aspects.

All data corroborated the fact that transmission by bite is not a homologous character, but a feature acquired independently by distinct lineages of trypanosomes. Apparently, other species can also be transmitted by bite, such as *T. (Megatrypanum) theileri*, whose development in ticks suggests that it can also be transmitted through both saliva and faeces (Burgdorfer, Schimidt & Hoogstraal, 1973).

All 34 isolates of *T. rangeli* and allied species investigated in this work were shown to be very closely related and were grouped tighter together in a robust cluster (Clade *T. rangeli*) by analysis of SSU rRNA sequences. Nevertheless, consistent levels of genetic diversity on both SSU and ITS sequences clearly separated these organisms into 4 major groups. Group A corresponds to that previously described for isolates from northwest South American countries by analysis of zymodemes, RAPD markers (Steindel *et al.* 1994; Maia da Silva *et al.* 2004) and mini-exon sequences (Grisard *et al.* 1999; Vallejo *et al.* 2003). Group B is formed exclusively by Brazilian isolates from Amazonia and was described before using spliced-leader gene markers (Silva *et al.* 1999) and RAPD analysis (Maia da Silva *et al.* 2004). Human isolates from Panama and El Salvador and a dog isolate from Colombia (RGB) were grouped with *T. leeuwenhoekii* (Group C). Likewise in all other previous studies based on zymodemes, RAPD patterns and mini-exon gene sequences (Steindel *et al.* 1994; Grisard *et al.* 1999; Maia da Silva *et al.* 2004), the isolate SC58 from Southern Brazil was clearly separated from all other taxa by both SSU and ITS gene polymorphism and ascribed to Group D.

There is not a significant degree of genetic polymorphism in either SSU or ITS sequences of the trypanosomes from wild mammals to justify their status as *T. rangeli*-like or as separate species allied to *T. rangeli*. However, there are enough molecular, epidemiological and biological data to assert the separation of isolates in high consistent and homogeneous phylogenetic groups, the taxonomic status of which needs further evaluation. For instance, *T. leeuwenhoekii* (from a sloth from Panama) was named as a different species of *Herpetosoma* allied to *T. rangeli* because it does not develop in *R. prolixus*,

whereas it could be transmitted by the bite of *R. pallescens* (Shaw, 1985). Likewise, some human isolates of *T. rangeli* from Panama and Colombia can be distinguished by their inability to develop in vector species other than *R. pallescens*, their sympatric triatomine species (Sousa & Dawson, 1976; Vallejo *et al.* 2003). *T. leeuwenhoekii* clustered tightly together with human *T. rangeli* isolates from Panama, El Salvador and Colombia in previous studies based on ribosomal and spliced leader sequences (Stevens *et al.* 1999) and on RAPD patterns (Maia da Silva *et al.* 2004).

Ribosomal (SSU and ITS) polymorphisms reported in this study and in a previous one based on RAPD markers (Maia da Silva *et al.* 2004) and on SL sequences (Silva *et al.* 1999; Maia da Silva *et al.* in preparation) disclosed 4 groups of *T. rangeli*. Thus, Clade *T. rangeli* is a complex taxon, constituted by different populations which appear to have evolved with non-random association of independent molecular markers. All groups contain members from different host species (human, domestic or sylvatic mammals), without any vertebrate host-species association. *T. rangeli* from sylvatic mammals can be very closely related to human isolates, suggesting that the same parasite circulates among humans, sylvatic and domestic animals and triatomines. Our data demonstrated that despite some geographical distance determination of segregation patterns, other factor(s) than geographical isolation are also determining these groups.

Sympatric triatomine species are excellent vectors for *T. rangeli* isolates, whereas non-sympatric species present different infection rates of salivary glands and can be even completely unsuitable as vectors (Sousa & Dawson, 1976; D'Alessandro & Saraiva, 1992; Machado *et al.* 2001). Vector and isolate parameters modulate the haemocoelic *T. rangeli* invasion and its survival in different vector species (Gomes *et al.* 1999). These data indicate a complex parasite–vector relationship and suggest co-evolution of *T. rangeli* populations with their local vector species. Recent analysis of Colombian *T. rangeli* isolates based on kDNA markers suggested either clonal evolution or speciation of *T. rangeli* populations in their triatomine vectors (Vallejo *et al.* 2003).

Taken together, biological and molecular features have now been gathered to demonstrate that *T. rangeli*, *T. rangeli*-like and allied species from distinct host and geographical origin can be tightly clustered together in the *T. rangeli* clade. In contrast, data are still insufficient to make definitive interpretations about evolutionary relationships among genetic groups within this clade. This would require comparison of more isolates from different vertebrate and triatomine species, from widespread geographical origins. New molecular markers and biological differences must be investigated before we can make definitive statements concerning both inter- and

intra-specific variations and the determining factors of segregation patterns within *T. rangeli* and related taxa.

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